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# Bioassay Guided Isolation of Active Compounds from *Alchemilla **barbatiflora*** Juz.

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**Abstract:** The aerial parts of *Alchemilla* L. species (Rosaceae) are used internally as diuretic, laxative, tonic and externally for wound healing in Turkish folk medicine. Antioxidant effects of the extracts, fractions and isolated compounds from the aerial parts of *A. barbatiflora* Juz. were investigated with following methods: 1,1-diphenyl-2-picryl-hydrazyl (DPPH), and superoxide radical scavenging (SOD), phosphomolibdenum-reducing antioxidant power (PRAP), ferric-reducing antioxidant power (FRAP) assays. In addition, tyrosinase, α-glucosidase and acetylcholinesterase inhibition activities of samples were analyzed. The methanol extract from the aerial parts of plant was consecutively fractionated into four subextracts; *n*-hexane, chloroform, and remaining water extracts. Further studies were carried out on the most active water subextract and the fractions obtained from water subextract with column chromatography. Phytochemical studies on active fractions of the water subextract led to the isolation of seven metabolites including catechin (**1**) and a catechin dimer; procyanidin B3 (**2**), a flavonol glucuronide; quercetin-3-*O*- *β*-D-glucuronic acid (miquelianin) (**3**) with flavonoid glycosides; quercetin-3-*O*- *β*-D-galactoside (hyperoside) (**4**), quercetin-3-*O*- *β*-D-arabinoside (guaiaverin) (**5**), kaempferol-3-*O*-*β*-D-xylopyranoside (**6**) and kaempferol-3-*O*-(6″-coumaroyl-*β*-D-glycoside) (tiliroside) (**7**). Their structures were elucidated by spectral techniques (1D and 2D NMR). The experimental data verified that procyanidin B3 displayed remarkable enzyme inhibitory activity among the whole isolated compounds.

**Keywords:** Antioxidant; α-Glucosidase; Rosaceae; tyrosinase. 

## 1. Introduction

The genus *Alchemilla* L. (Rosaceae) is represented by more than 1000 species all around the World and 77 species in the flora of Turkey [1-3]. *Alchemilla* genus is known with a valuable medical plant; *Alchemilla vulgaris* (Lady's mantle) which is used in phytotherapy because of its astringent, diuretic and antispasmodic properties [4]. Some members of the genus *Alchemilla* are reported to be used against slow metabolism related diseases, dysmenorrhoea, menorrhagia and as antiinflammatory

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and for wound treating worldwide [5,6]. Moreover, aerial parts of some *Alchemilla* species have been used in Turkish folk medicine for bronchitis, rheumatoid arthritis and as diuretic, constipant, tonic, emmenagogue, menstrual regulator [7-9]. *Alchemilla barbatiflora* Juz. which is native to the Caucasus, is reported to be boiled and drunk against liver inflammation, dyspnea, gynecological diseases in the Black Sea Region of Turkey [10]. Previous phytochemical studies on *Alchemilla* species revealed that the genus is rich in flavonoids, phenolic acids, tannins and triterpenes, besides, quercetin and kaempferol glycosides were reported to be characteristic for the genus [11-15]. Biological activities of the *Alchemilla* species are mainly focused on *A. vulgaris* of which aqueous methanol extract has been reported to possess antioxidant [16], acetylcholinesterase inhibitory [17], antiproliferative [18] activities. Our research aimed to identify the chemical constituents from the aerial parts of *A. barbatiflora*, responsible for the activity. To the best of our knowledge, the present work is the first report about the chemical constituents and biological activity of *A. barbatiflora*.

## 2. Materials and Methods

### *2.1. Plant Materials*

The aerial parts of *A. barbatiflora* were collected from Trabzon, Zigana Pass and identified by Dr. Gülin Renda. A voucher specimen was deposited in Hacettepe University Faculty of Pharmacy Herbarium (HUEF 15018).

### *2.2. Extraction*

Aerial parts of the plant were dried and powdered. The powdered plant material (440 g) was extracted thrice with methanol:water (90:10) mixture by stirring at 40 °C for 6 h. After filtration, the methanol extracts were combined and evaporated to dryness in vacuum (not exceeding 40 °C) to give crude methanol extract (CME) (117.81 g).

### *2.3. Fractionation of the Crude Methanol Extract*

The crude methanol extract was dispersed in water and extracted with *n*-hexane (4×300 mL) in a separatory funnel. Combined *n*-hexane phases were evaporated under reduced pressure to yield '*n*-hexane sub-extract' (HSE) (2.10 g). Then the residual water phase was further fractionated with chloroform (4×500 mL). Chloroform phases as well as remaining aqueous phases were evaporated to dryness under reduced pressure to yield "chloroform sub-extract" (CSE) (16.91 g), and "water sub-extract" (WSE) (95.81 g), respectively. The dry sub-extracts were stored in amber flasks at 4°C.

### *2.4. Isolation and Determination of the Compounds from Water Sub-extract*

75 grams of the water sub-extract was fractionated over a polyamide column (VLC, 100 g), eluting with gradient MeOH/$H_2O$ mixtures (0-100 %) to afford 6 main fractions (Frs.A-F). Fraction E (3.12 g) was subjected to vacuum liquid chromatography (VLC) over reversed-phase stationary phase (LiChroprep C18) and elution with $H_2O$, followed by increasing concentrations of MeOH in $H_2O$ mixtures (0 → 100 % MeOH, in steps of 10 % of MeOH, each 100 mL, fraction volumes 100 mL) as eluent, yielded 5 fractions (Frs.E1-E5).

Fraction E1 (1.5343 g) was subjected to a silica gel column (150 g) chromatography using $CHCl_3$/MeOH/$H_2O$ with increasing polarity (90:10:0.5 to 50:50:5 mixtures) which yielded **1** (7.3 mg) and **2** (5.7 mg). Fr. E2 (0.368 g) was purified by Sephadex LH-20 CC using MeOH to yield **3** (24.9 mg). Fr.E3 (0.7821 g) was rechromatographed over a silica gel column (90 g) eluting stepwise with $CHCl_3$/MeOH/$H_2O$ (90:10:0.5 to 50:50:5) mixtures and was further purified by Sephadex LH-20 CC using MeOH to obtain pure **4** (11.0 mg) and **5** (3.3 mg). Fr. E4 (0.0996 g) was subjected to silica gel (12 g) CC with $CHCl_3$/MeOH (98:2 to 90:10) and further purified by Sephadex LH-20 CC using MeOH to afford pure **6** (2.5 mg). Purification of Fraction E5 (0.1180 g) by preparative TLC gave **7** (5.9 mg) (Figure 1).

### *2.5. Biological Activity Tests*

#### *2.5.1. DPPH Radical Scavenging Assay*

The *in vitro* antioxidant activities of the samples were investigated with DPPH free radical scavenging assay method [19]. 1 mL of the assay mixture was prepared with methanolic DPPH solution (0.1 mM) and various concentrations of samples. The absorbances of the samples ($A_{sample}$) were measured at 517 nm after incubation in dark at room temperature for 30 min. Control was consist of assay mixture without samples (control absorbance, $A_{control}$) while positive control was consist of gallic acid. Formula 1 was used to calculate free radical scavenging effect.

$$\text{Formula 1: Scavenging effects (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

#### *2.5.2. Superoxide Radical Scavenging Assay*

A non-enzymatic superoxide radical ($O_2^{-\bullet}$) generation assay was used to determine the superoxide scavenging activity of the samples [20]. The assay mixture which has a total volume of 1 mL, was consist of riboflavin, NBT, methionine, EDTA, and the test samples (phosphate buffer, pH 7.8). The absorbance of the samples ($A_{sample}$) was calculated at 560 nm after lighting up 10 min with a fluorescent lamp at 30 ºC. Control was consist of assay mixture without samples (control absorbance, $A_{control}$) finally the results were calculated using the Formula 1.

#### *2.5.3. Phosphomolibdenum-reducing Antioxidant Power (PRAP) Assay*

In order to perform PRAP assays of the samples, various concentrations of samples were mixed with a 10 % phosphomolybdic acid solution in ethanol (w/v) [21]. Solutions were subsequently subjected to incubation at 80 °C for 30 min. After incubation, the absorbances were measured at 600 nm and compared to references.

#### *2.5.4. Ferric-reducing Antioxidant Power (FRAP ) Assay*

FRAP of samples was determined using the method described by Oyaizu [22,23]. Various concentrations of the samples and BHA as the standard were added to phosphate buffer (pH 6.6) and 10 % (w/v) $K_3[Fe(CN)_6]$. The mixtures were incubated at 50 °C for 20 min and then 10 % TCA was added. After vigorous shaking, the solutions were mixed with distilled water and 0.15 % $FeCl_3$. The mixtures were incubated for 30 min at room temperature in the dark. After incubation, the absorbance was measured at 700 nm. The FRAP of samples was expressed as butylated hydroxyanisole equivalents (BHAE) per g of dry weight sample.

#### *2.5.5. Tyrosinase Inhibition Assay*

The method defined by Masuda was used to examine tyrosinase inhibition [24]. The samples at various concentrations, 250 U/mL tyrosinase and pH 6.8 phosphate buffer solutions (100mM) were separately added in a 96-well microplate. The reaction was started by inserting 3 mM L-DOPA and the absorbance was measured at 475 nm. Kojic acid was the positive control and formula 2 was used to measure the tyrosinase inhibition percentage. The inhibitory concentration of 50 % of tyrosinase ($IC_{50}$) values was measured from the graphic of the percentage inhibition in front of extract concentrations.

$$\text{Formula 2: Inhibition (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

#### *2.5.6. α-Glucosidase Inhibition Assay*

α-Glucosidase inhibition was examined by the method of da Silva Pinto [25]. Acarbose was the reference drug. All samples at various concentrations and 0.5 U/mL α-glucosidase enzyme were separately included in a 96-well microplate and the final mixtures were incubated at 25 ˚C for 15 min. After incubation, 5 mM p-nitrophenyl-α-glucopyranoside was added and incubated at 25 ˚C for 10

min. The absorbance was calculated at 405 nm and formula 2 was used to measure α-glucosidase inhibition percentage.

### *2.5.7. Acetylcholinesterase (AChE) Inhibition Assay*

Acetylcholinesterase (AChE) inhibition was examined using the method described by Ingkaninan [26, 27]. Galantamine was used as the positive control. The mixtures of Tris-HCl buffer (pH 8.00), DTNB (in buffer), AChE and all samples at various concentrations were incubated at 25 °C added in a 96-well microplate for 15 min. After incubation, 25 µL of 15 mM AChI was included to the mixture and waited for 5 min at room temperature. The absorbances were calculated at 412 nm and the formula 2 was used to measure the AChE inhibition.

### *2.6. Statistical Analysis*

The experiments were repeated three times and results were expressed as the mean ± standard deviation (SD). The statistical analysis was performed with SPSS 15.0 for Windows and Microsoft Excel for Windows 10. The differences among the extracts were evaluated by one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. $P < 0.05$ was considered statistically significant.

## 3. Results and Discussion

### *3.1. Biological Activities*

In this study, the antioxidant activities of the extracts and fractions from the aerial parts of *A. barbatiflora* were analyzed by using DPPH, SOD, PRAP, FRAP assays. DPPH and SOD radicals are utilized for the determination of the radical-scavenging ability of antioxidants [28]. The results of the DPPH and SOD radical scavenging assays are presented in Table 1. CME showed remarkable DPPH and SOD radical scavenging activities with 83.44 ± 0.02 % and 83.34 ± 0.02 % at 125 µg/mL. Among the tested sub-extracts, WSE showed the best results with 83.06 ± 0.02 %, 96.08 ± 0.11 % and 97.17 ± 0.23 % at 125, 250 and 500 µg/mL, respectively, for DPPH scavenging activities. HSE displayed moderate DPPH scavenging activities as compared to GA. In SOD assay at 125 µg/mL, WSE had significant SOD radical scavenging activities with 81.07 ± 0.02 % when compared to SOD enzyme (Table 1).

Reducing power activities of samples were determined by PRAP and FRAP assays. As seen in Table 2, WSE had higher absorbance than CME in PRAP assay. In FRAP assay, the result of CME was found as 44.32 ± 0.83 mg BHAE/g extract while the result of WSE was as 93.46 ± 1.36 mg BHAE/g extract (Table 2).

**Table 1.** Radical scavenging activities (% inhibition) of extracts, sub-extracts and fractions

| | DPPH | | | SOD | | |
|---|---|---|---|---|---|---|
| | **125 µg/mL** | **250 µg/mL** | **500 µg/mL** | **125 µg/mL** | **250 µg/mL** | **500 µg/mL** |
| CME | 83.44 ± 0.02[a] | 93.90 ± 0.14 | 95.35 ± 0.06 | 83.34 ± 0.02 | 85.83 ± 0.10 | ND |
| HSE | 18.6 ± 0.85 | 28.89 ± 0.01 | 59.62 ± 0.06 | 9.80 ± 0.10 | ND | ND |
| CSE | 67.17 ± 0.03 | 91.11 ± 0.01 | ND | 12.84 ± 0.08 | 22.34 ± 0.13 | 42.73 ± 0.12 |
| WSE | 83.06 ± 0.02 | 96.08 ± 0.11[b] | 97.17 ± 0.23[b] | 81.07 ± 0.02 | ND | ND |
| A | 83.06 ± 0.39 | ND | ND | 79.86 ± 0.08 | ND | ND |
| B | 85.25 ± 0.09 | ND | ND | 73.09 ± 0.09 | ND | ND |
| C | 85.32 ± 0.02 | ND | ND | 83.14 ± 0.13 | ND | ND |
| D | 87.14 ± 0.04 | 96.08 ± 0.09 | ND | 84.61 ± 0.53[b] | 87.64 ± 0.08 | 90.09 ± 0.05 |
| E | 88.15 ± 0.07 | 97.08 ± 0.14[b] | ND | 83.34 ± 0.31 | 86.54 ± 0.09 | ND |
| F | 86.47 ± 0.08 | ND | ND | 79.43 ± 0.06 | ND | ND |
| GA | 95.18 ± 0.02 | 95.36 ± 0.43 | 98.75 ± 0.01 | - | - | - |
| SOD | | | | 88.08 ± 0.11 | 93.25 ± 0.14 | 95.30 ± 0.31 |

CME: Crude methanol extract, HSE: *n*-hexane sub-extract, CSE: chloroform sub-extract, WSE: water sub-extract ND:No Data [a]Values expressed are means ± SD, [b]($p < 0.05$)

In this context, tyrosinase, α-glucosidase and acetylcholinesterase inhibition activities of the extracts, fractions and isolated compounds from the aerial parts of *A. barbatiflora* were investigated and the results were presented in Tables 3, 4 and 5. In tyrosinase inhibition assay, CME had moderate inhibition with 22.30 ± 0.77 %, 33.63 ± 1.16 %, and 43.36 ± 1.96 % at 125, 250 and 500 µg/mL, respectively. Among the tested sub-fractions, HSE and CSE did not show inhibition against tyrosinase enzyme, but WSE indicated tyrosinase inhibition with 45.59 ± 0.51 % at 500 µg/mL. In the α-glucosidase inhibition assay, CME showed significant inhibition against α-glucosidase with 90.56 ± 0.15 % and 95.36 ± 0.28 % at 125 and 250 µg/mL, respectively. WSE indicated remarkable inhibition against α-glucosidase while HSE and CSE did not show any inhibition against enzyme. In the acetylcholinesterase inhibition assay, CME inhibited the enzyme with 11.66 ± 0.27 %, 20.54 ± 2.08 % and 34.81 ± 0.51 % at 125, 250 and 500 µg/mL, respectively. However, WSE showed low inhibition activities with 4.88 ± 0.22 % at 125 µg/mL. The results revealed that WSE showed statistically significant activity at all of the tests performed. Therefore, the WSE was subjected to further detailed studies. The fractions (A-F) which were prepared from WSE were tested within the same system.

**Table 2.** Reducing power activities of extracts, sub-extracts and fractions

| | PRAP | | FRAP |
|---|---|---|---|
| | **125 µg/mL** | **250 µg/mL** | **mg BHAE/g extract** |
| CME | 0.932 ± 0.002[a] | 1.280 ± 0.004 | 44.32 ± 0.83 |
| HSE | 0.355 ± 0.003 | 0.612 ± 0.006 | ND |
| CSE | 0.640 ± 0.001 | 0.802 ± 0.003 | 15.76 ± 0.25 |
| WSE | 1.158 ± 0.001 | 1.516 ± 0.006 | 93.46 ± 1.36 |
| A | 1.190 ± 0.003 | 3.198 ± 0.013 | 158.14 ± 0.90 |
| B | 1.932 ± 0.005 | 3.806 ± 0.016[b] | 168.23 ± 1.33 |
| C | 2.241 ± 0.001 | ND | 223.33 ± 2.44 |
| D | 3.516 ± 0.006 | ND | 227.75 ± 1.50 |
| E | 3.519 ± 0.017[b] | ND | 227.93 ± 0.94 |
| F | 2.538 ± 0.002 | ND | 225.93 ± 0.47 |
| QE | 1.891 ± 0.002 | 3.501 ± 0.018 | - |

The results of PRAP assay was given as absorbance and FRAP assay as mg BHAE/g extract. CME: Crude methanol extract, HSE: *n*-hexane sub-extract, CSE: chloroform sub-extract, WSE: water sub-extract ND:No Data [a]Values expressed are means ± SD, [b]($p < 0.05$)

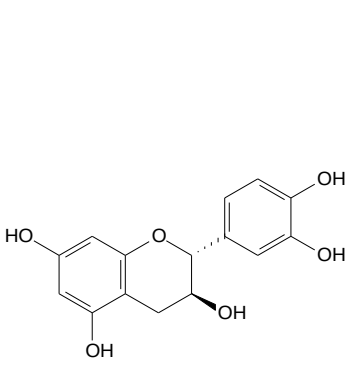
**Table 3.** Tyrosinase and α–glucosidase inhibition activities of extracts, sub-extracts and fractions (%inhibition)

| | Tyrosinase | | | α-glucosidase | | |
|---|---|---|---|---|---|---|
| | **125 µg/mL** | **250 µg/mL** | **500 µg/mL** | **125 µg/mL** | **250 µg/mL** | **500 µg/mL** |
| CME | 22.30 ± 0.77[a] | 33.63 ± 1.16 | 43.36 ± 1.96[b] | 90.56 ± 0.15 | 95.36 ± 0.28 | ND |
| HSE | ND | ND | ND | ND | ND | ND |
| CSE | ND | ND | ND | ND | ND | ND |
| WSE | 27.91 ± 0.39 | 36.05 ± 2.66[b] | 45.59 ± 0.51[b] | 95.29 ± 0.05[b] | 96.73 ± 0.19[b] | ND |
| A | 10.72 ± 1.20 | 33.50 ± 0.58 | 45.75 ± 0.75 | 80.64 ± 1.39[b] | 92.06 ± 0.99[b] | 95.87 ± 0.75 |
| B | 35.03 ± 1.59 | 49.82 ± 1.42 | 64.54 ± 0.40 | 80.22 ± 1.15 | ND | ND |
| C | 28.84 ± 2.38 | 55.38 ± 2.12 | ND | 81.48 ± 0.61 | ND | ND |
| D | 38.98 ± 1.59 | 63.18 ± 1.48 | ND | 84.48 ± 0.55 | 91.98 ± 0.33 | ND |
| E | 30.02 ± 0.74 | 62.25 ± 2.25 | 90.50 ± 2.37 | 86.79 ± 0.20 | 93.87 ± 0.42[b] | ND |
| F | 27.79 ± 0.76 | 49.46 ± 1.38[b] | ND | 80.10 ± 0.98 | ND | ND |
| Kojic Acid | 84.40 ± 0.24 | 87.34 ± 0.02 | 90.36 ± 0.15 | - | 75.12 ± 0.12 | 86.97 ± 1.27 |
| Acarbose | - | - | - | 66.88 ± 0.15 | 95.36 ± 0.28 | ND |

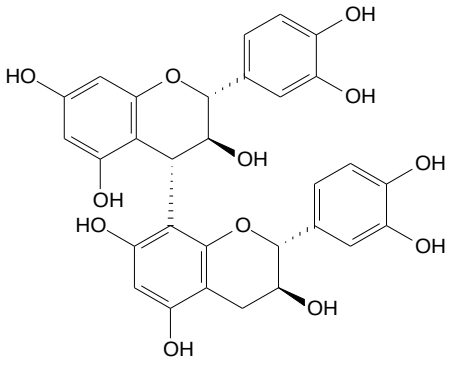
CME: Crude methanol extract, HSE: *n*-hexane sub-extract, CSE: chloroform sub-extract, WSE: water sub-extract ND:No Data [a]Values expressed are means ± SD, [b]($p < 0.05$)

Fraction E showed the best results with 88.15 ± 0.07 % and 97.08 ± 0.14 % for DPPH scavenging assay and 83.34 ± 0.31 % and 86.54 ± 0.09 % for SOD radical scavenging assay. Fraction E showed higher absorbance with 3.519 ± 0.017 among the tested new fractions and QE in PRAP assay. Fraction E from WSE showed the best results with 227.93 ± 0.94 mg BHAE/g extract (Table 1

and 2). At 500 µg/mL, Fraction E from WSE showed significant tyrosinase inhibition with 90.50 ± 2.37 % when compared to kojic acid. Fraction A from WSE showed the highest α-glucosidase inhibition with 95.87 ± 0.75 % at 500 µg/mL whereas fraction E from WSE indicated the best results with 93.87 ± 0.42 % at 250 µg/mL.



**1**



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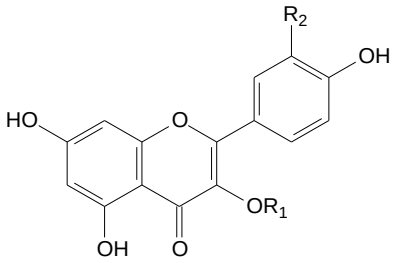

| | $R_1$ | $R_2$ |
|---|---|---|
| **3** | *β*-D-glucoronic acid | -OH |
| **4** | *β*-D-galactose | -OH |
| **5** | *β*-D-arabinose | -OH |
| **6** | *β*-D-xylose | -H |
| **7** | 6-coumaroyl-*β*-D-glucose | -H |

**Figure 1.** Structures of compounds **1-7** isolated from *A. barbatiflora*

Due to promising results obtained from fraction E, the isolation studies were performed on this fraction. Hence, phytochemical studies on fraction E led to the isolation of seven previously reported metabolites. The identification of the isolated compounds was carried out by spectroscopic analysis including 1D- and 2D- NMR ($^1$H, $^{13}$C, COSY, HSQC and HMBC) spectroscopy. The known compounds were identified as catechin (**1**) [29], procyanidin B3 (**2**) [30], quercetin-3-*O*-glucuronic acid (miquelianin) (**3**) [31], quercetin-3-*O*-galactoside (hyperoside) (**4**) [32], quercetin-3-*O*-arabinoside (guaiaverin) (**5**) [33], kaempferol-3-*O*-*β*-D-xylopyranoside (**6**) [34], and kaempferol-3-*O*-(6″-E-coumaroyl-*β*-D-glycoside) (tiliroside) (**7**) [35] by comparison of their spectroscopic data with those of published values.

*Catechin (**1**):* $C_{15}H_{13}O_6$; $^1$H NMR ($CD_3OD$, 600 MHz) δ : 6.835 (1H, d, *J* = 2.0 Hz), 6.760 (1H, d, *J* = 8.1 Hz), 6.716 (1H, dd, *J* = 8.1, 2.0 Hz), 5.924 (1H, d, *J* = 2.3 Hz), 5.851 (1H, d, *J* = 2.3 Hz), 4.560 (1H, d, *J* = 7.5 Hz), 3.971 (1H, ddd, *J* = 8.2, 7.5, 5.4 Hz), 2.847 (1H, dd, *J* = 16.1, 5.4 Hz), 2.502 (1H, dd, *J* = 16.1, 8.2 Hz).

*Procyanidin B3 (**2**):* $C_{30}H_{25}O_{12}$; Mixture of rotational isomers $^1$H NMR ($CD_3OD$, 600 MHz) major isomer δ : 6.737 (1H, d, *J* =2.0 Hz), 6.677 (1H, d, *J* =8.2 Hz), 6.673 (1H, d, *J* =8.2 Hz), 6.587 (1H, d, *J* =2.0 Hz), 6.471 (1H, dd, *J* =8.2, 2.0 Hz), 6.255 (1H, ddd, *J* =8.2, 2.0, 0.7 Hz), 6.072 (1H, s), 5.888 (1H, d, *J* =2.4 Hz), 5.788 (1H, d, *J* =2.4 Hz), 4.540 (1H, dd, *J* =7.4, 0.7 Hz), 4.411 (1H, d, *J* =7.9 Hz), 4.352 (1H, dd, *J* =9.7, 7.9 Hz), 4.256 (1H, d, *J* =9.7 Hz), 3.791 (1H, ddd, *J* =8.1, 7.4, 5.5 Hz), 2.763

(1H, dd, *J* =16.3, 5.5 Hz), 2.487 (1H, dd, *J* =16.3, 8.1 Hz). minor isomer δ : 6.956 (1H, d, *J* =2.0 Hz), 6.955 (1H, d, *J* =2.0 Hz), 6.832 (1H, dd, *J* =8.2, 2.0 Hz), 6.823 (1H, dd, *J* =8.1, 2.0 Hz), 6.767 (1H, d, *J* =8.1 Hz), 6.762 (1H, d, *J* =8.2 Hz), 5.941 (1H, s), 5.839 (1H, d, *J* =2.4 Hz), 5.810 (1H, d, *J* =2.4 Hz), 4.744 (1H, d, *J* =7.3 Hz), 4.525 (1H, dd, *J* =9.2, 8.0 Hz), 4.498 (1H, d, *J* =8.0 Hz), 4.361 (1H, d, *J* =9.2 Hz), 4.073 (1H, ddd, *J* =7.7, 7.3, 5.4 Hz), 2.821 (1H, dd, *J* =16.2, 5.4 Hz), 2.585 (1H, dd, *J* =16.2, 7.7 Hz). $^{13}$C NMR ($CD_3OD$, 151 MHz) major isomer δ : 158.63, 157.13 (2C), 155.87, 155.64, 154.88, 146.07, 145.79, 145.61, 145.47, 132.63, 131.85, 120.61, 119.87, 116.42, 116.20, 116.05, 115.51, 108.16, 107.18, 102.25, 97.32, 96.87, 96.05, 83.95, 82.47, 73.69, 68.91, 38.59, 28.78. minor isomer δ : 158.62, 157.44, 157.32, 155.86, 155.77, 154.98, 146.37, 146.14 (2C), 146.08, 132.41, 132.16, 121.02, 120.16, 116.16, 116.14, 115.91, 115.20, 108.34, 107.13, 100.49, 97.55, 97.50, 96.23, 84.10, 82.96, 73.67, 68.56, 38.55, 28.49

*Hyperoside (**4**):* $C_{21}H_{20}O_{12}$; $^1$H NMR ($CD_3OD$, 600 MHz) δ : 7.842 (1H, d, *J* =2.2 Hz), 7.585 (1H, dd, *J* =8.5, 2.2 Hz), 6.861 (1H, d, *J* =8.5 Hz), 6.377 (1H, d, *J* =2.1 Hz), 6.186 (1H, d, *J* =2.1 Hz), 5.147 (1H, d, *J* =7.8 Hz), 3.853 (1H, dd, *J* =3.4, 1.2 Hz), 3.822 (1H, dd, *J* =9.7, 7.8 Hz), 3.644 (1H, dd, *J* =11.2, 6.0 Hz), 3.560 (1H, dd, *J* =9.7, 3.4 Hz), 3.556 (1H, dd, *J* =11.2, 6.5 Hz), 3.474 (1H, ddd, *J* =6.5, 6.0, 1.2 Hz). $^{13}$C NMR ($CD_3OD$, 151 MHz) δ :179.41 (C-4), 166.72 (C-7), 163.01 (C-5), 158.68 (C-2), 158.49 (C-8a), 149.99 (C-4'), 145.82 (C-3'), 135.75 (C-3), 122.92 (C-6'), 122.87 (C-1'), 117.74 (C-2'), 116.08 (C-5'), 105.47 (C-4a, C1''), 99.91 (C-6), 94.82 (C-8), 77.18 (C-5''), 75.11 (C-3''), 73.18 (C-2''), 70.03 (C-4''), 61.94 (C-6'').

*Guaiaverin (**5**):* $C_{20}H_{18}O_{11}$; $^1$H NMR ($CD_3OD$, 600 MHz) δ : 7.743 (1H, d, *J* =2.2 Hz), 7.577 (1H, dd, *J* =8.5, 2.2 Hz), 6.867 (1H, d, *J* =8.5 Hz), 6.362 (1H, d, *J* =2.1 Hz), 6.177 (1H, d, *J* =2.1 Hz), 5.139 (2H, d, *J* =6.6 Hz), 3.898 (2H, dd, *J* =8.4, 6.6 Hz), 3.823 (2H, m), 3.813 (2H, m), 3.642 (2H, m), 3.443 (2H, m).

**Table 4.** Acetylcholinesterase inhibition activities (% inhibition) of extracts, sub-extracts and fractions

| | **Acetylcholinesterase** | | |
|---|---|---|---|
| μg/mL | 125 | 250 | 500 |
| CME | 11.66 ± 0.27[a] | 20.54 ± 2.08 | 34.81 ± 0.51[b] |
| HSE | ND | ND | ND |
| CSE | ND | ND | ND |
| WSE | 4.88 ± 0.22 | ND | ND |
| A | ND | ND | ND |
| B | ND | ND | ND |
| C | ND | 14.38 ± 0.61 | 25.17 ± 0.38 |
| D | 11.08 ± 0.71 | 21.63 ± 0.83 | 35.08 ± 0.99[b] |
| E | ND | 8.17 ± 1.66 | 15.82 ± 0.57 |
| F | ND | ND | ND |
| Galantamine | 89.28 ± 0.20 | 92.11 ± 0.18 | 95.19 ± 0.17 |

CME: Crude methanol extract HSE: *n*-hexane sub-extract, CSE: chloroform sub-extract, WSE: water sub-extract ND:No Data [a]Values expressed are means ± SD, [b]($p < 0.05$)

**Table 5.** Tyrosinase and α-glucosidase inhibition activities of isolated compounds

| | Tyrosinase (μM) | α-glucosidase (μM) |
|---|---|---|
| Compounds | $IC_{50}$ | $IC_{50}$ |
| Catechin | ND | > 250.00 |
| Procyanidin B3 | 56.58 ± 2.22 | 241.60 ± 4.21 |
| Quercetin-3-*O*-glucuronic acid | > 250.00 | > 250.00 |
| Quercetin-3-*O*-galactoside | 77.46 ± 1.85 | > 250.00 |
| Quercetin-3-*O*-arabinoside | 127.94 ± 4.56 | > 250.00 |
| Kaempferol-3-*O*-*β*-D-xylopyranoside | > 250.00 | > 250.00 |
| Kaempferol-3-*O*-*β*-D-glycoside | 50.95 ± 1.59 | > 250.00 |
| Kojic Acid | 16.40 ± 0.38 | - |
| Acarbose | - | 57.97 ± 0.23 |

ND:No Data [a]Values expressed are means ± SD, [b]($p < 0.05$)

Because of the better results obtained at tyrosinase inhibition and α-glucosidase inhibition assays, all of the isolated compounds were analyzed for tyrosinase inhibition and α-glucosidase inhibition. Among the isolated compounds, kaempferol-3-*O*-β-D-glycoside had the lowest $IC_{50}$ values and highest activity for tyrosinase inhibition with 50.95 ± 1.59 µM. In addition, $IC_{50}$ values of procyanidin B3 and quercetin-3-*O*-galactoside were found to be 56.58 ± 2.22 µM and 77.46 ± 1.85 µM, respectively. In addition, procyanidin B3 was found to have 241.60 ± 4.21 µM of $IC_{50}$ values for α-glucosidase inhibition.

Flavonoids and tannins have been reported from various *Alchemilla* species therewithal quercetin and kaempferol glycosides were reported to be characteristic for the genus [13]. It can be deduced that the chemical composition of the *A. barbatiflora* is similar with the other *Alchemilla* species studied previously. Among the isolated compounds; catechin was previously isolated from *A. mollis* [12] and *A. speciosa* [36], tiliroside was isolated from *A. achtarowii* [13] and *A. mollis* [37], while miquelianin and guaiaverin were isolated from *A. xanthochlora* [14, 38] and *A. achtarowii* [13]. As well as hyperoside was reported to be present in *A. procerrima*, *A. hirtipedicellata*, *A. sericata* [11]. However, catechin dimer; procyanidin B3 is being reported for the first time from the genus *Alchemilla*. It was isolated from *Rosa laevigata* [39] and *Potentilla parviflora* [40] which are the members of the same family Rosaceae.

Antioxidant [16, 37, 41-43] activity of the aerial parts of some *Alchemilla* species have been investigated only with DPPH assay and the species were found to possess high activity. Tyrosinase inhibitory activity of *A. vulgaris* extracts was found to be very small whereas the high value of acetylcholinesterase inhibitory activity of the ethanolic extract of A. vulgaris has been reported [17].

## 4. Conclusions

In this study the secondary metabolites and biological activity of *A. barbatiflora*, on which no chemical or biological studies had been performed before, has been investigated. Our findings stand out *A. barbatiflora* as an important source of tannins and flavonoid glycosides with many biological activities. Even though the water subextract showed significant enzymatic inhibition, only compound 2 showed higher activity among the other compounds. Other enzyme inhibitors, that could not be isolated, should be responsible for the observed activity of the water subextract. Additionally, the compound procyanidin B3 which was obtained for the first time from this genus with this study may contribute to the chemotaxonomy of the genus *Alchemilla*.

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